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(54) Title: ETHANOL PROCESS

(57) Abstract: The present invention relates to an improved ethanol production process wherein the viscosity of liquefied mash and/or the Thin Stillage and/or condensate and/or syrup of evaporated Thin Stillage are reduced by addition of an effective amount of thinning enzyme selected from the group consisting of alpha-amylase, xylanase, xyloglucanase, cellulase, pectinase, or a mixture thereof.

ETHANOL PROCESS

FIELD OF THE INVENTION

The present invention relates to an improved process of producing ethanol; the use of enzyme activity for thinning the liquefied whole grain mash and the Thin Stillage and/or condensate thereof (evaporated Thin Stillage) and/or syrup thereof; a composition comprising alpha-amylase, xylanase, xyloglucanase, cellulase, pectinase, or a mixture thereof, suitably reducing the viscosity of mash and of Thin Stillage and/or condensate thereof and/or syrup thereof.

BACKGROUND OF THE INVENTION

Ethanol has widespread application as an industrial chemical, gasoline additive or straight liquid fuel. As a fuel or fuel additive, ethanol dramatically reduces air emissions while improving engine performance. As a renewable fuel, ethanol reduces national dependence on finite and largely foreign fossil fuel sources while decreasing the net accumulation of carbon dioxide in the atmosphere. Fermentation processes are used for the production of ethanol. There is exists a vast number of disclosure concerning production of alcohol by fermentation, among which are, e.g., US 5,231,017 and CA 1,143,677.

There is a need for further improvement of ethanol manufacturing processes.

BRIEF DESCRIPTION OF THE DRAWING

Fig. 1 schematically shows an ethanol production process of the invention.

DESCRIPTION OF THE INVENTION

The invention relates to an improved process of producing ethanol as will be described below.

Ethanol production

In ethanol production processes the starting raw material is in a preferred embodiment of the invention whole grain. However, the side-products from starch conversion processes may also be used. A wide variety of starch containing whole grain crops may be used as raw material including: corn (maize), milo, potato, cassava, sorghum, wheat, and barley.

The main process steps can be separated into the following main process stages:

- Milling,
- Liquefaction,
- Saccharification,

- Fermentation,
- Distillation.

The individual process steps of alcohol production may be performed batch wise or as a continuous flow. For the invention processes where one or more process step(s) is(are) performed batch wise or one or more process step(s) is(are) performed as a continuous flow, are equally contemplated. Thus contemplated are also processes where the fermentation step is performed as a continuous flow. The cascade process is an example of a process where one or more process step(s) is(are) performed as a continuous flow and as such contemplated for the invention. Further information on the cascade process and other ethanol processes can be found in, e.g., "The Alcohol Textbook" Eds. T.P. Lyons, D.R. Kesall and J.E. Murtagh. Nottingham University Press 1995.

Milling

The whole grain is milled in order to open up the structure and allowing for further processing. Two processes can be used: wet and dry milling. Preferred for ethanol production, at least according to the present invention, is dry milling where the whole kernel is milled and used in the remaining part of the process. Wet milling gives a good separation of germ and meal (starch granules and protein) and is with a few exceptions applied at locations where there is a parallel production of syrups. Both dry and wet milling is well known in the art of ethanol production. Both dry and wet milling is contemplated for the present invention.

Liquefaction

In an embodiment of the liquefaction step of the invention, milled gelatinized starch whole grain raw material is broken down (hydrolyzed) into maltodextrins (dextrins) mostly of a DP higher than 4. The hydrolysis may be carried out by acid treatment or enzymatically by alpha-amylase. Acid hydrolysis is used on a limited basis. The raw material is in one embodiment milled whole grain or a side stream from starch processing.

In an embodiment the invention, enzymatic liquefaction is carried out as a three-step hot slurry process. The slurry is heated to between 60-95°C, preferably 80-85°C, and the enzyme(s) is (are) added to initiate liquefaction (thinning). Then the slurry is jet-cooked at a temperature between 95-140°C, preferably 105-125°C to complete gelanitization of the slurry. Then the slurry is cooled to 60-95°C and more enzyme(s) is(are) added to finalize hydrolysis. The liquefaction process is carried out at pH 4.5-6.5, in particular at a pH between 5 and 6. Milled and liquefied whole grain is also known as mash.

Saccharification

To produce low molecular sugars DP_{1-2} that can be metabolized by yeast, the maltodextrin from the liquefaction must be further hydrolyzed. The hydrolysis is typically done enzymatically in the presence of a glucoamylase (AMG). An alpha-glucosidase and/or an acid alpha-amylase may also be present.

A full saccharification step may last up to 72 hours, however, it is common only to do a pre-saccharification of typically 40-90 minutes and then complete saccharification during fermentation (SSF). Saccharification may be carried out at temperatures from 30-65°C, in particular around 60°C, and at a pH in the range between 4-5, especially around pH 4.5.

Fermentation

Yeast typically from *Saccharomyces* spp., in particular *Saccharomyces cerevisiae* or bakers yeast, is added to the mash and the fermentation is ongoing for 24-96 hours, such as 35-60 hours. The temperature is between 26-34°C, in particular at about 32°C, and at pH 3-6, in particular around pH 4-5.

The most widely used process is a simultaneous saccharification and fermentation (SSF) process where there is no holding stage for the saccharification, meaning that yeast and enzyme is added together. When doing SSF it is common to introduce a pre-saccharification step at a temperature above 50°C, just prior to the fermentation.

Following the fermentation the mash is distilled to extract the ethanol. The ethanol obtained according to the process of the invention may be used as, e.g., fuel ethanol; drinking ethanol, i.e., potable neutral spirits; or industrial ethanol.

Distillation

The alcohol is separated from the fermented mash and purified. Ethanol with a purity of up to about 96 vol.% ethanol is obtained.

By-products from distillation

The aqueous by-product (Whole Stillage) from the distillation process is separated into two fractions, for instance by centrifugation: 1) Wet Grain (solid phase), and 2) Thin Stillage (Supernatant).

The Wet Grain fraction is dried, typically in a drum dryer. The dried product is referred to as "Distillers Dried Grain", and can be used as animal feed.

The Thin Stillage fraction may be evaporated providing two fractions:

- condensate fraction of 4-6% DS (mainly of starch, proteins, and cell wall components), and

- Syrup fraction, mainly consisting of limit dextrins and non fermentable sugars, which may be introduced into a dryer together with the Wet Grain (from the Whole Stillage separation step) to provide a product referred to as "Distillers Dried Grain", which can be used as animal feed.

"Whole Stillage" is the term used in the art for the side-product (main product is ethanol) coming from the distillation of fermented mash (see Fig. 1).

"Thin Stillage" is the term used in the art for the supernatant of the centrifugation of the Whole Stillage. Typically, the Thin Stillage contains 4-6% DS (mainly starch and proteins) and has a temperature of about 60-90°C. Thin Stillage is viscous and difficult to handle. Thin Stillage is normally kept in a holding tank for up to a few hours before recycling to the slurry tank (see Fig. 1).

Process of the invention

In the first aspect the invention relates to a process of producing ethanol, wherein liquefied whole grain mash is thinned by treatment with an efficient amount of enzyme activity selected from the group consisting of alpha-amylase, xylanase, xyloglucanase, cellulase, pectinase, and mixtures thereof.

In the second aspect the invention relates to a process of producing ethanol, wherein the Thin Stillage and/or condensate and/or syrup of evaporated Thin Stillage is thinned by treatment with an efficient amount of enzyme activity selected from the group consisting of alpha-amylase, xylanase, xyloglucanase, cellulase, pectinase, and mixtures thereof.

In an embodiment the treated or untreated Thin Stillage and/or condensate and/or syrup of evaporated Thin Stillage is recycled to the liquefaction step, in particular to the milled whole grain slurry.

A specific ethanol production process (system) is shown in Fig. 1.

In an embodiment the invention relates to a process for the production of ethanol which comprise the following steps:

a) *milling whole grain;*

b) liquefying gelatinised whole grain in the presence of an alpha-amylase;

c) saccharifying the liquefied material in the presence of a glucoamylase;

d) fermentation with a micro-organism;

e) *distillation of the fermented and saccharified material, providing two fractions:* 1) alcohol fraction, and 2) Whole Stillage fraction;

(f1) recovering ethanol; optionally the recovered ethanol is further refined;

(f2) separating the Whole Stillage into two fractions of: 1) Wet Grain (solid phase), and 2) Thin Stillage (*supernatant*);

(g1) the Wet Grain fraction is dried to provide a protein containing product referred to as "Distillers Dried Grain", which is used as an animal feed product;

(g2) the Thin Stillage may be evaporated providing two fractions: 1) Condensate fraction, and 2) Syrup fraction (mainly consisting of limit dextrin and non-fermentable sugars), which is introduced into a (drum) dryer together with the Wet Grain fraction (from the Whole Stillage separation step) to provide a product referred to as "Distillers Dried Grain", which is used as animal feed.

In a preferred embodiment of the invention the liquefaction step comprising the following sub-steps:

b1) the hot slurry is heated to between 60-95°C, preferably 80-85°C, and at least an alpha-amylase is added;

b2) the slurry is jet-cooked at a temperature between 95-140°C, preferably 105-125°C to complete gelanitization of the slurry;

b3) the slurry is cooled to 60-95°C and more alpha-amylase is added to finalize hydrolysis.

Typically the DS% (dry solid percentage) in the slurry tank (containing milled whole grain) is in the range from 1-60%, in particular 10-50%, such as 20-40%, such as 25-35%.

The liquefaction process is in an embodiment carried out at pH 4.5-6.5, in particular at a pH between 5 and 6.

The thinning of the mash is preferably carried out or initiated as early in the process as possible.

Therefore, in an embodiment of the invention the thinning enzyme(s) is(are) added to the liquefied mash during or after the secondary liquefaction step, Alternatively the thinning enzyme(s) is(are) added to the mash at the mash cooling step.

In another embodiment of the invention the thinning enzyme(s) are added to the mash during pre-saccharification, saccharification, fermentation, or SSF.

Steps (c) and (d) (i.e., saccharification and fermentation) may be carried out either simultaneously or separately/sequential. Further, after step (d) an optional ethanol recovery step may be added.

After fermentation the fermented and according to the invention thinned mash is distilled to provide an ethanol fraction as described above. The ethanol fraction may optionally be refined further.

The Thin Stillage from step (f2) and/or condensate and/or syrup of Thin Stillage from step (g2) may be recycled to the liquefaction step, in particular to the slurry containing the milled whole grain to be jet cooked.

Optionally the Thin Stillage from step (f2) or the condensate from step (g2) is subjected to an effective amount of enzyme activity selected from the group consisting of alpha-amylase, xylanase, xyloglucanase, cellulase, pectinase, and mixtures thereof. According to the invention the enzyme treatment is preferably performed in the holding tank.

The term "thinning enzyme(s)" used above means that the liquefied mash and/or the Thin Stillage and/or condensate and/or syrup of Thin Stillage is subjected to an effective amount of enzyme selected from the group of alpha-amylase, xylanase, xyloglucanase, cellulase, pectinase activity, or mixtures thereof.

In an embodiment the treated thin stillage and/or condensate and/or syrup is recycled to the liquefaction step b), in particular to the (gelatinized) milled whole grain slurry to be jet cooked.

In another embodiment Thin Stillage is not recycled, but the condensate stream and/or the syrup of evaporated Thin Stillage is recycled to the liquefaction step, in particular to the slurry containing the milled whole grain to be jet cooked.

Before recycling, the Thin Stillage and/or the condensate and/or the syrup of evaporated Thin Stillage is subjected to an effective amount of an enzyme activity selected from the group consisting of alpha-amylase, xylanase, xyloglucanase, cellulase, pectinase, and mixtures thereof. Mixtures contemplated include the following combination: alpha-amylase and xylanase activity; alpha-amylase and cellulase activity; alpha-amylase and pectinase activity; xylanase and cellulase activity; xylanase and pectinase activity; cellulase and pectinase activity; alpha-amylase, xylanase and cellulase activity; alpha-amylase, xylanase and pectinase activity; xylanase, cellulase and pectinase activity; alpha-amylase, cellulase and pectinase activity.

In another embodiment additional thinning enzymes, in particular hemicellulose degrading enzymes, such as xyloglucanases are added to the whole grain slurry. The viscosity of the whole grain slurry is, in part, a result of the hemicellulose contribution to the overall viscosity. When the viscosity is reduced using effective hemicellulose-degrading enzymes like xylanases, xyloglucanases the structure of dry milled corn is altered, more closely resembling a corn that has been extremely and efficiently fine milled to a smaller particle size. Various enzymatic systems have been tested showing that it is possible to process corn at a higher dry solid content, >40% DS vs. current 35-38% DS, while maintaining an acceptable viscosity for further processing.

The above-mentioned (combinations) of enzyme activities may be in particular any of the below listed.

Enzyme activities used for treating Whole Grain Mash and/or Thin Stillage

Alpha-amylase

Liquefaction of the gelatinized milled whole grain slurry may be performed in the presence of an alpha-amylase derived from a micro-organism or a plant. Preferred alpha-amylases are of fungal or bacterial origin. *Bacillus* alpha-amylases (often referred to as "Termamyl-like alpha-amylases"), variant and hybrids thereof, are specifically contemplated according to the

invention. Well-known Termamyl-like alpha-amylases include alpha-amylase derived from a strain of *B. licheniformis* (commercially available from Novozymes A/S as Termamyl™), *B. amyloliquefaciens*, and *B. stearothermophilus* alpha-amylase. Other Termamyl-like alpha-amylases include alpha-amylase derived from a strain of the *Bacillus* sp. NCIB 12289, NCIB 12512, NCIB 12513 or DSM 9375, all of which are described in detail in WO 95/26397, and the alpha-amylase described by Tsukamoto et al., Biochemical and Biophysical Research Communications, 151 (1988), pp. 25-31. In the context of the present invention a Termamyl-like alpha-amylase is an alpha-amylase as defined in WO 99/19467 on page 3, line 18 to page 6, line 27. Contemplated variants and hybrids are described in WO 96/23874, WO 97/41213, and WO 99/19467. Contemplated alpha-amylase derived from a strain of *Aspergillus* includes *Aspergillus oryzae* and *Aspergillus niger* alpha-amylases.

Specifically contemplated variants include: *Bacillus stearothermophilus* alpha-amylase variants disclosed in WO 99/19467.

Commercial alpha-amylase products and products containing alpha-amylases include TERMAMYL™ SC, FUNGAMYL™, LIQUOZYME™ SC and SAN™ SUPER, (from Novozymes A/S) and DEX-LO™, SPEZYME™ AA, and SPEZYME™ DELTA AA (from Genencor Int.)

Other contemplated alpha-amylases include the KSM-K36 alpha-amylase disclosed in EP 1,022,334 and deposited as FERM BP 6945, and the KSM-K38 alpha-amylases disclosed in EP 1,022,334, and deposited as FERM BP-6946. Also variants thereof are contemplated, in particular the variants disclosed in Danish patent application no. PA 2000 11533 (from Novozymes A/S).

Treatment of the Thin Stillage and/or condensate and/or syrup of evaporated Thin Stillage according to the process of the invention may be carried out in the presence of an effective amount of any of the above-mentioned alpha-amylases.

Xylanase

Treatment of the Thin Stillage and/or condensate and/or syrup of evaporated Thin Stillage according to the process of the invention may be carried out in the presence of an effective amount of any of the following xylanases. Xylanase activity may be derived from any suitable organism, including fungal and bacterial organisms, such as *Aspergillus*, *Disporotrichum*, *Penicillium*, *Neurospora*, *Fusarium* and *Trichoderma*.

Examples of suitable xylanases include xylanases derived from *H. insolens* (WO 92/17573; *Aspergillus tubigensis* (WO 92/01793); *A. niger* (Shei et al., 1985, Biotech. and Bioeng. Vol. XXVII, pp. 533-538, and Fournier et al., 1985, Bio-tech. Bioeng. Vol. XXVII, pp. 539-546; WO 91/19782 and EP 463 706); *A. aculeatus* (WO 94/21785).

In a specific embodiment the xylanase is Xylanase II disclosed in WO 94/21785.

Contemplated commercially available xylanase include SHEARZYME®, BIOFEED WHEAT®, and PULPZYME™ HC (from Novozymes A/S) and SPEZYME® CP (from Genencor Int).

Xyloglucanase

The xyloglucanases, used in accordance with the invention may be any xyloglucanase, particularly of microbial origin, such as from a filamentous fungus or bacteria. Specific examples of fungal xyloglucanases include the xyloglucanases obtainable from strains belonging to the genus *Malbranchea*, such as the species *Malbranchea cinnamomea* as disclosed in WO 0112794A1, or from strains belonging to the genus *Aspergillus*, such as from the species *Aspergillus aculeatus* as disclosed in WO 94/14953. Specific examples of bacterial xyloglucanases include the xyloglucanases obtainable from strains of the genus *Paenibacillus* such as the species *P. pabuli* or *P. polymyxa* as disclosed in WO 0162903A1, or from strains of the genus Bacillus such as from the species *Bacillus licheniformis* and *Bacillus agaradhaerens* as disclosed in WO 99/02663, or from strains of the genus *Jonesia*.

Cellulase

The cellulase, used in accordance with the invention, may be any cellulase, in particular of microbial origin, in particular fungal or bacterial origin such as a cellulase derivable from a strain of a filamentous fungus (e.g., *Aspergillus*, *Trichoderma*, *Humicola*, *Fusarium*). Specific examples of cellulases include the endoglucanase (endo-glucanase I) obtainable from *H. insolens* and further defined by the amino acid sequence of fig. 14 in WO 91/17244 and the 43 kD *H. insolens* endoglucanase described in WO 91/17243.

Commercially available cellulases, which may be used include CELLUCLAST®, CELLUZYME® (available from Novozymes A/S), SPEZYME® CP (available from Genencor, USA) and ROHAMENT® 7069 W (available from Röhm, Germany).

Pectinase

The pectinase may be any pectinase, in particular of microbial origin, in particular of bacterial origin, such as a pectinase derived from a species within the genera *Bacillus*, *Clostridium*, *Pseudomonas*, *Xanthomonas* and *Erwinia*, or of fungal origin, such as a pectinase derived from a species within the genera *Aspergillus*, in particular from a strain within the species *A. niger* and *A. aculeatus*. Contemplated commercially available pectinases include PECTINEX® and BIOPREP™ (available from Novozymes A/S).

Enzyme activities used during saccharification or SSF

Glucoamylase

The saccharification step or the simultaneous sac-charification and fermentation step (SSF) may be carried out in the presence of a glucoamylase derived from a micro organism or a plant. Preferred is glucoamylase of fungal or bacterial origin selected from the group consisting of *Aspergillus niger* glucoamylase, in particular *A. niger* G1 or G2 glucoamylase (Boel et al. (1984), EMBO J. 3 (5), p. 1097-1102), or variants thereof, such as disclosed in WO 92/00381 and WO 00/04136; the *A. awamori* glucoamylase (WO 84/02921), *A. oryzae* (Agric. Biol. Chem. (1991), 55 (4), p. 941-949), or variants or fragments thereof.

Other contemplated *Aspergillus* glucoamylase variants include variants to enhance the thermal stability: G137A and G139A (Chen et al. (1996), Prot: Engng. 9, 499-505); D257E and D293E/Q (Chen et al. (1995), Prot. Engng. 8, 575-582); N182 (Chen et al. (1994), Biochem. J. 301, 275-281); disulphide bonds, A246C (Fierobe et al. (1996), Biochemistry, 35, 8698-8704; and introduction of Pro residues in position A435 and S436 (Li et al. (1997), Protein Engng. 10, 1199-1204. Furthermore Clark Ford presented a paper on Oct 17, 1997, ENZYME ENGINEERING 14, Beijing/China Oct 12-17, 97, Abstract num-ber: Abstract book p. 0-61. The abstract suggests mutations in positions G137A, N20C/A27C, and S30P in an *Aspergillus awamori* glucoamylase to improve the thermal stability. Other glucoamylases include *Talaromyces* glucoamylases, in particular derived from *Talaromyces emersonii* (WO 99/28448), *Talaromyces leycettanus* (US patent no. Re. 32,153), *Talaromyces duponti*, *Talaromyces thermopiles* (US patent no. 4,587,215). Bacterial glucoamylases contemplated include glucoamylases from the genus Clostridium, in particular *C. thermoamylolyticum* (EP 135,138), and *C. thermohydrosulfuricum* (WO 86/01831).

Commercial products include SAN™, SUPER™ and AMG™ E (from Novozymes).

Protease

Addition of protease(s) in the saccharification step or SST step increase(s) the FAN (Free amino nitrogen) level and increase the rate of metabolism of the yeast and further gives higher fermentation efficiency.

Suitable proteases include fungal and bacterial proteases. Preferred proteases are acidic proteases, i.e., proteases characterized by the ability to hydrolyze proteins under acidic conditions below pH 7.

Suitable acid fungal proteases include fungal proteases derived from *Aspergillus*, *Mucor*, *Rhizopus*, *Candida*, *Coriolus*, *Endothia*, *Enthomophtra*, *Irpex*, *Penicillium*, *Sclerotiumand Torulopsis*. Especially contemplated are proteases derived from *Aspergillus niger* (see, e.g., Koaze et al., (1964), Agr. Biol. Chem. Japan, 28, 216), *Aspergillus saitoi* (see, e.g., Yoshida, (1954) J. Agr. Chem. Soc. Japan, 28, 66), *Aspergillus awamori* (Hayashida et al., (1977) Agric. Biol.

Chem., 42(5), 927-933, *Aspergillus aculeatus* (WO 95/02044), or *Aspergillus oryzae,* such as the pepA protease; and acidic proteases from *Mucor pusillus* or *Mucor miehei.*

Bacterial proteases, which are not acidic proteases, include the commercially available products Alcalase® and Neutrase® (available from Novozymes A/S).

Additional enzyme

One or more additional enzymes may also be used during saccharification or SSF. Additional enzymes include pullulanase and phytase.

Microorganism used for fermentation

The micro organism may be a fungal organism, such as yeast or bacteria. Examples of filamentous fungi include strains of *Penicillium* sp. Preferred organisms for ethanol production is yeasts. Preferred yeast according to the invention is baker's yeast, also known as *Saccharomyces cerevisiae.*

Composition of the invention

Finally the invention related to a composition comprising an alpha-amylase, xylanase, xyloglucanase, cellulase, pectinase, and mixtures thereof. The alpha-amylase, xylanase, cellulase, and pectinase activity may have the above-mentioned origin.

MATERIAL & METHODS

Determination of Viscosity

The mash is heated to a temperature of 50-70°C, depending on the treatment. Following treatment viscosity is measured using a Haake VT02 rotation based viscosimeter. The unit of viscosity is centipois (cps), which is proportionally related to the viscosity level.

Determination of Alpha-Amylase Activity (KNU)

1. Phadebas assay

Alpha-amylase activity is determined by a method employing Phadebas® tablets as substrate. Phadebas tablets (Phadebas® Amylase Test, supplied by Pharmacia Diagnostic) contain a cross-linked insoluble blue-colored starch polymer, which has been mixed with bovine serum albumin and a buffer substance and tabletted.

For every single measurement one tablet is suspended in a tube containing 5 ml 50 mM Britton-Robinson buffer (50 mM acetic acid, 50 mM phosphoric acid, 50 mM boric acid, 0.1 mM $CaCl_2$, pH adjusted to the value of interest with NaOH). The test is performed in a water bath at the temperature of interest. The alpha-amylase to be tested is diluted in x ml of 50 mM Britton-

Robinson buffer. 1 ml of this alpha-amylase solution is added to the 5 ml 50 mM Britton-Robinson buffer. The starch is hydrolyzed by the alpha-amylase giving soluble blue fragments. The absorbance of the resulting blue solution, measured spectrophotometrically at 620 nm, is a function of the alpha-amylase activity.

It is important that the measured 620 nm absorbance after 10 or 15 minutes of incubation (testing time) is in the range of 0.2 to 2.0 absorbance units. In this absorbance range there is linearity between activity and absorbance (Lambert-Beer law). The dilution of the enzyme must therefore be adjusted to fit this criterion. Under a specified set of conditions (temperature, pH, reaction time, buffer conditions) 1 mg of a given alpha-amylase will hydrolyze a certain amount of substrate and a blue colour will be produced. The measured absorbance is directly proportional to the specific activity (activity/mg of pure alpha-amylase protein) of the alpha-amylase in question under the given set of conditions.

2. Alternative method

Alpha-amylase activity is alternatively determined by a method employing the PNP-G7 substrate. PNP-G7 which is a abbreviation for p-nitrophenyl-alpha,D-maltoheptaoside is a blocked oligosaccharide which can be cleaved by an endo-amylase. Following the cleavage, the alpha-glucosidase included in the kit digest the substrate to liberate a free PNP molecule which has a yellow colour and thus can be measured by visible spectophometry at wavelength Lambda=405nm (400-420 nm). Kits containing PNP-G7 substrate and alpha-glucosidase is manufactured by Bohringer-Mannheim (cat. No. 1054635).

To prepare the substrate one bottle of substrate (BM 1442309) is added to 5 ml buffer (BM1442309). To prepare the alpha-glucosidase one bottle of alpha-glucosidase (BM 1462309) is added to 45 ml buffer (BM1442309). The working solution is made by mixing 5 ml alpha-glucosidase solution with 0.5 ml substrate.

The assay is performed by transforming 20 microL enzyme solution to a 96 well microtitre plate and incubating at 25°C. 200 microL working solution, 25°C is added. The solution is mixed and pre-incubated 1 minute and absorption is measured every 15 seconds over 3 minutes at OD 405 nm.

The slope of the time dependent absorption-curve is directly proportional to the specific activity (activity per mg enzyme) of the alpha-amylase in question under the given set of conditions.

Determination of Acid Amylolytic Activity (FAU)

One Fungal Alpha-Amylase Unit (1 FAU) is defined as the amount of enzyme, which breaks down 5.26 g starch (Merck Amylum solubile Erg. B.6, Batch 9947275) per hour at Novozymes' standard method for determination of alpha-amylase based upon the following standard conditions:

Substrate	Soluble starch
Temperature	37°C
pH	4.7
Reaction time	7-20 minutes

A detailed description of Novozymes´ method is available on request.

Determination of acid alpha-amylase activity (AFAU)

Acid alpha-amylase activity is measured in AFAU (Acid Fungal Alpha-amylase Units), which are determined relative to an enzyme standard.

The standard used is AMG 300 L (wild type *A. niger* G1 AMG sold by Novozymes A/S). The neutral alpha-amylase in this AMG falls after storage at room temperature for 3 weeks from approx. 1 FAU/mL to below 0.05 FAU/mL.

The acid alpha-amylase activity in this AMG standard is determined in accordance with AF 9 1/3 (Novo method for the determination of fungal alpha-amylase). In this method, 1 AFAU is defined as the amount of enzyme, which degrades 5.260 mg starch dry matter per hour under standard conditions.

Iodine forms a blue complex with starch but not with its degradation products. The intensity of colour is therefore directly proportional to the concentration of starch. Amylase activity is determined using reverse colorimetry as a reduction in the concentration of starch under specified analytic conditions.

	Alpha-amylase	
Starch + Iodine	→	Dextrins + Oligosaccharides
	40°C, pH 2.5	
Blue/violet		t=23 sec. Decolouration

<u>Standard conditions/reaction conditions:</u> (per minute)

Substrate:	starch, approx. 0.17 g/L
Buffer:	Citrate, approx. 0.03 M
Iodine (I_2):	0.03 g/L
$CaCl_2$:	1.85 mM
pH:	2.50 ± 0.05
Incubation temperature:	40°C

Reaction time:	23 seconds
Wavelength:	Lambda=590nm
Enzyme concentration:	0.025 AFAU/mL
Enzyme working range:	0.01-0.04 AFAU/mL

Further details can be found in EB-SM-0259.02/01 available on request from Novozymes A/S, which folder is hereby incorporated by reference.

Determination Of Glucoamylase Activity (AGU)

The Amyloglucosidase Unit (AGU) is defined as the amount of enzyme, which hydrolyzes 1 micromole maltose per minute at 37°C and pH 4.3. A detailed description of the analytical method (AEL-SM-0131) is available on request from Novozymes A/S.

The activity is determined as AGU/ml by a method modified after (AEL-SM-0131, available on request from Novozymes A/S) using the Glucose GOD-Perid kit from Boehringer Mannheim, 124036. Standard: AMG-standard, batch 7-1195, 195 AGU/ml. 375 microL substrate (1% maltose in 50 mM Sodium acetate, pH 4.3) is incubated 5 minutes at 37°C. 25 microL enzyme diluted in sodium acetate is added. The reaction is stopped after 10 minutes by adding 100 microL 0.25 M NaOH. 20 microL is transferred to a 96 well microtitre plate and 200 microL GOD-Perid solution (124036, Boehringer Mannheim) is added. After 30 minutes at room temperature, the absorbance is measured at 650 nm and the activity calculated in AGU/ml from the AMG-standard.

Determination of Xylanase activity (FXU)

The endoxylanase activity is determined by an assay, in which the xylanase sample is incubated with a remazol-xylan substrate (4-O-methyl-D-glucurono-D-xylan dyed with Remazol Brilliant Blue R, Fluka), pH 6.0. The incubation is performed at 50°C for 30 min. The background of non-degraded dyed substrate is precipitated by ethanol. The remaining blue colour in the supernatant is determined spectrophotometrically at 585 nm and is proportional to the endoxylanase activity.

The endoxylanase activity of the sample is determined relatively to an enzyme standard.

The assay is further described in the publication AF 293.6/1-GB, available upon request from Novozymes A/S, Denmark.

Xyloglucanase assay (XyloU)

The xyloglucanase activity is measured using AZCL-xyloglucan from Megazyme, Ireland, (http://www.megazyme.com/purchase/index.html) as substrate.

A solution of 0.2 % of the blue substrate is suspended in a 0.1 M phosphate buffer pH 7.5 under stirring. The solution is distributed under stirring to 1.5 ml Eppendorf tubes (0.75 ml to each), 50 µl enzyme solution is added and they are incubated in an Eppendorp Thermomixer model 5436 for 20 minutes at 40°C with a mixing of 1200 rpm. After incubation the coloured solution is separated from the solid by 4 minutes centrifugation at 14,000 rpm and the absorbance of the supernatant is measured at 600 nm.

One XyloU unit is defined as the amount of enzyme resulting in an absorbance of 0.24 in a 1 cm cuvette at 600 nm.

Determination of Endo-Glucanase Units (ECU)

The ECU (endocellulose unit) is determined relatively to an enzyme standard. Endocellulase decomposes carboxylmethylcellulose, CMC. The prepared substrate solution contains 35 g/l CMC (Blanose Aqualon) in 0.1 M phosphate buffer at pH 7.5. The enzyme sample to be analysed is determined is dissolved in the same buffer.

0.15 ml standard enzyme solution or the unknown enzyme sample is placed in 10 ml test tubes. 5 ml CMC-substrate solution, preheated to 40°C, is added. The joint solution is mixed thoroughly, incubated for 30 minutes and placed in the viscometer.

The method is further described in details in AF302/1-GB available from Novozymes A/S upon request.

Determination of Endo-glucanase activity (EGU)

A substrate solution containing 34.0 g/l CMC (Blanose Aqualon) in 0.1 M phosphate buffer, pH 6.0 is prepared. The enzyme sample to be analysed is dissolved in the same buffer. 14 ml substrate solution and 0.5 ml enzyme solution are mixed and transferred to a vibration viscosimeter (e.g. MIVI 3000 available from Sofraser, France) thermostated at 40°C.

Endoglucanase unit (EGU) is determined as the ratio between the viscosity of the sample and the viscosity of a standard enzyme solution.

Determination of Cellulytic Activity (NCU)

The cellulytic activity is determined with carboxymethyl cellulose (CMC) as substrate.

One Cellulase Unit (NCU) is defined as the amount of enzyme which, under standard conditions (i.e., at pH 4.80; 0.1 M acetate buffer; 10 g/l Hercules CMC type 7 LFD as substrate; an incubation temp. of 40.0°C; an incubation time of 20 minutes; and an enzyme concentration of approximately 0.041 NCU/ml) forms an amount of reducing carbohydrates equivalent to 1 micro mol glucose per minute.

A folder AF 187.2/1 describing this analytical method in more detail is available upon request to Novozymes A/S, Denmark, which folder is hereby included by reference.

Claims

1. A process of producing ethanol, wherein liquefied whole grain mash is thinned by treatment with an efficient amount of enzyme activity selected from the group consisting of alpha-amylase, xylanase, xyloglucanase, cellulase, pectinase, and mixtures thereof.

2. The process of claim 1 where the ethanol producing method is as shown in Fig. 1.

3. A process of producing ethanol, wherein the process sequentially comprises the following steps:

a) milling whole grain;

b) liquefying the gelatinised milled whole grain, in the presence of an alpha-amylase;

c) saccharifying the liquefied material in the presence of a glucoamylase;

d) fermentation with a micro-organism;

e) distillation of fermented and saccharified material, providing an ethanol fraction,

wherein the liquefied mash is thinned by subjection to an effective amount enzyme activity selected from the group consisting of alpha-amylase, xylanase, xyloglucanase, cellulase, pectinase, or a mixture thereof.

4. The process of any of claims 1-3, wherein the liquefaction step comprising the following sub-steps:

b1) the hot slurry is heated to between 60-95°C, preferably 80-85°C, and at least an alpha-amylase is added;

b2) the slurry is jet-cooked at a temperature between 95-140°C, preferably 105-125°C to complete gelanitization of the slurry;

b3) the slurry is cooled to 60-95°C and more alpha-amylase is added to finalize hydrolysis.

5. A process of producing ethanol, wherein Thin Stillage and/or condensate and/or syrup of evaporated Thin Stillage is thinned by treatment with an efficient amount of enzyme activity selected from the group consisting of alpha-amylase, xylanase, xyloglucanase, cellulase, pectinase, and mixtures thereof.

6. The process of claim 5 where the ethanol producing method is as shown in Fig. 1.

7. A process of producing ethanol, wherein the process sequentially comprises the following steps:

a) milling whole grain;

b) liquefying the gelatinised milled whole grain, in the presence of an alpha-amylase;

c) saccharifying the liquefied material in the presence of a glucoamylase;

d) fermentation with a micro-organism;

e) distillation of fermented and saccharified material, providing two fraction: 1) an alcohol fraction and 2) a Whole Stillage fraction;

f) separating the Whole Stillage into two fractions: 1) Wet Grain fraction, and 2) Thin Stillage;

g) optionally the Thin Stillage is evaporated to provide two fractions: 1) Condensate and 2) Syrup,

wherein the Thin Stillage and/or condensate and/or syrup from step (g) is subjected to an effective amount of enzyme activity selected from the group consisting of alpha-amylase, xylanase, xyloglucanase, cellulase, pectinase.

8. The process of any of claims 5-7, wherein the treated Thin Stillage and/or condensate and/or syrup is recycled to the liquefaction step b), in particular to the milled whole grain slurry.

9. The process of any of claims 5-8, wherein the Wet Grain fraction from step g) is dried to provide a protein containing product;

10. A process of producing ethanol, wherein

a. The liquefied whole grain mash

and

b. The Thin Stillage and/or condensate and/or syrup of Thin Stillage

is thinned by treatment with an efficient amount of enzyme activity selected from the group consisting of alpha-amylase, xylanase, xyloglucanase, cellulase, pectinase, and mixtures thereof.

11. The process of any of claims 1 and 5 where the ethanol producing method is as shown in Fig. 1.

12. The process of any of claims 1-11 wherein the whole grain is dry milled.

13. The process of any of claims 1-12, wherein the liquefaction process is carried out at pH 4.5-6.5, in particular at a pH between 5 and 6.

14. The process of any of claims 1-13, wherein the DS% in the slurry to be liquefied is between 20-40%.

15. The process of any of claims 1-14, wherein the thinning enzyme(s) are added to the liquefied mash in or after the secondary liquefaction step.

16. The process of any of claims 1-15, wherein the thinning enzyme(s) are added to the mash at the mash cooling step.

17. The process of any of claims 1-16, wherein the thinning enzyme(s) are added to the mash during pre-saccharification, saccharification, fermentation or SSF.

18. The process of any of claims 1-17, wherein whole grain is dry milled.

19. The process of any of claims 1-17, wherein whole grain is wet milled.

20. The process of any of claims 1-19, wherein the alpha-amylase is derived from a strain of the genus *Bacillus* or a strain of *Aspergillus*, in particular the alpha-amylase is a Termamyl-like alpha-amylase or KSM-K36 or KSM-K38, or a variant thereof.

21. The process of any of claims 1-20, wherein the xylanase is derived from *Aspergillus*, in particular *Aspergillus aculeatus*.

22. The process of any of claims 1-21 wherein the cellulase is the 43 kD endoglucanase (cellulase) from *Humicola insolens*.

23. The process of any of claims 1-22, wherein the microorganism is a yeast, such as *Saccharomyces cerevisae*.

24. The process of any of claims 1-23, wherein the whole grain is selected from the group comprising corn (maize), milo, potato, cassava, sorghum, wheat, and barley, in particular corn.

25. The process of any of claims 1-24, wherein the milled whole grain slurry is liquefied to DE of 5-20, in particular 8-12.

26. The process of any of claims 1-25, wherein the saccharification and fermentation step is carried out either simultaneously or separately/sequentially.

26. Use of an alpha-amylase, xylanase, xyloglucanase, cellulase, pectinase, or a mixture thereof for thinning of liquefied mash.

27. A composition comprising an alpha-amylase, xylanase, xyloglucanase, cellulanase, pectinase, or a mixture thereof.

28. The process of any of claims 1 to 27 where one or more process step(s) is(are) performed batch wise and/or one or more process step(s) is(are) performed as a continuous flow.

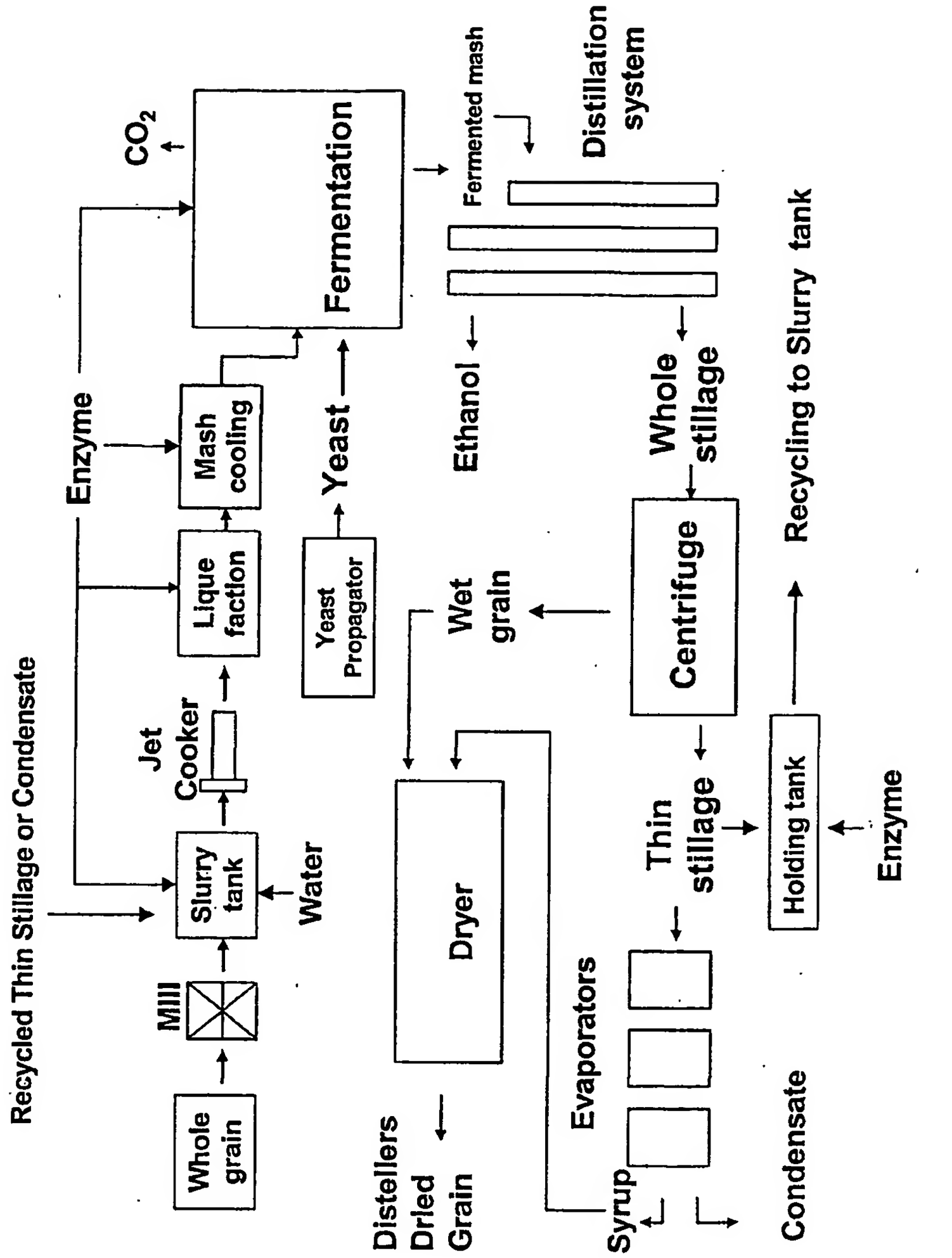
Recycled Thin Stillage or Condensate
Enzyme
CO_2
Fermentation
Fermented mash
Distillation system
Ethanol
Mash cooling
Lique faction
Yeast Propagator
Yeast
Jet Cooker
Slurry tank
Water
Mill
Whole grain
Wet grain
Centrifuge
Whole stillage
Thin stillage
Holding tank
Enzyme
Recycling to Slurry tank
Dryer
Distellers Drled Grain
Evaporators
Syrup
Condensate

Fig. 1

INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK 01/00709

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12P7/06 C12N9/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12P C12N C12G

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
PAJ, BIOSIS, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 231 017 A (FISH JOHN J ET AL) 27 July 1993 (1993-07-27) column 1, line 39 - line 44 column 3, line 65 -column 4, line 2 ---	1-28
X	US 4 474 883 A (YAMAMOTO TAKEHIKO ET AL) 2 October 1984 (1984-10-02) claims 1-4,26-34 ---	1-28
X	DD 236 109 A (SWS STAMMBETRIEB VEB BAERENSIE) 28 May 1986 (1986-05-28) page 4, line 12 - line 25 --- -/--	1-28

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 January 2002	22. 02. 2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	ekström nils

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INTERNATIONAL SEARCH REPORT

Int Application No
PCT/DK 01/00709

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE BIOSIS [Online] BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; PREV198579048970, BELDMAN G ET AL: "Application of cellulase EC-3.2.1.4 and pectinase EC-3.2.1.15 from fungal origin for liquefaction and saccharification of biomass" XP002902260 Enzyme and microbial technology abstract ---	26-28
X	WO 95 04134 A (GENENCOR INT) 9 February 1995 (1995-02-09) abstract ---	26-28
A	US 5 023 176 A (DUCROO PAUL) 11 June 1991 (1991-06-11) abstract; claims 1,2 ---	1-28
A	US 5 187 081 A (COUDERC RENE ET AL) 16 February 1993 (1993-02-16) examples 3,4 ---	1-28
A	WO 96 13600 A (GENENCOR INT) 9 May 1996 (1996-05-09) claims 1,7 -----	1-30

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/DK 01/00709

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5231017 A	27-07-1993	AT 170217 T	15-09-1998
		AU 661702 B2	03-08-1995
		AU 2155692 A	30-12-1992
		BR 9206015 A	27-12-1994
		CA 2102896 A1	18-11-1992
		CN 1069768 A ,B	10-03-1993
		CN 1231926 A	20-10-1999
		DE 69226772 D1	01-10-1998
		DE 69226772 T2	14-01-1999
		DK 585374 T3	25-05-1999
		EP 0585374 A1	09-03-1994
		JP 7509118 T	12-10-1995
		WO 9220777 A1	26-11-1992
US 4474883 A	02-10-1984	JP 1632455 C	26-12-1991
		JP 2060317 B	14-12-1990
		JP 57152888 A	21-09-1982
		AU 552943 B2	26-06-1986
		AU 8137882 A	23-09-1982
		BR 8201351 A	25-01-1983
		DE 3207676 A1	28-10-1982
		DK 109882 A	15-09-1982
		NL 8200606 A	01-10-1982
DD 236109 A	28-05-1986	DD 236109 A1	28-05-1986
WO 9504134 A	09-02-1995	WO 9504134 A1	09-02-1995
US 5023176 A	11-06-1991	AT 61402 T	15-03-1991
		CA 1280704 A1	26-02-1991
		CN 86108167 A ,B	05-08-1987
		DE 3677920 D1	11-04-1991
		DK 578586 A	04-06-1987
		EP 0227159 A2	01-07-1987
		FI 864897 A ,B,	04-06-1987
		IE 59503 B	09-03-1994
		JP 1966205 C	25-08-1995
		JP 6097984 B	07-12-1994
		JP 62215380 A	22-09-1987
		PT 83862 A ,B	01-01-1987
US 5187081 A	16-02-1993	FR 2627507 A1	25-08-1989
		AT 106447 T	15-06-1994
		CA 1336175 A1	04-07-1995
		DE 68915568 D1	07-07-1994
		DE 68915568 T2	12-01-1995
		DK 76189 A	19-08-1989
		EP 0329559 A1	23-08-1989
		JP 1247086 A	02-10-1989
		JP 2848620 B2	20-01-1999
WO 9613600 A	09-05-1996	AU 4013295 A	23-05-1996
		CA 2203811 A1	09-05-1996
		EP 0788551 A1	13-08-1997
		FI 971781 A	25-04-1997
		WO 9613600 A1	09-05-1996